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FGFs, Wnts and BMPs mediate induction of VEGFR-2 (*Quek-1*) expression during avian somite development

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Abstract

Regulation of VEGFR-2 (Quek1) is an important mechanism during blood vessel formation. In the paraxial mesoderm, *Quek1* expression is restricted to the lateral portion of the somite and later to sclerotomal cells surrounding the neural tube. By implanting FGF 8b/8c or SU 5402 beads into the paraxial mesoderm, we show that FGF8 in addition to BMP4 from the intermediate mesoderm (IM) is a positive regulator of VEGFR-2 (*Quek1*) expression in the quail embryo. The expression of *Quek1* in the medial somite half is normally repressed by the notochord and Sfrps-expression in the neural tube. Over-expression of Wnt 1/3a also results in an up-regulation of *Quek1* expression in the somites. We also show that up-regulation of FGF8/Wnt 1/3a leads to an increase in the number of endothelial cells, whereas inhibition of FGF and Wnt signaling by SU 5402 and Sfrp-2 results in a loss of endothelial cells. Our results demonstrate that the regulation of *Quek1* expression in the somites is mediated by the cooperative actions of BMP4, FGF8 and Wnt-signaling pathways.

Keywords: Quek1; VEGFR-2; Fibroblast growth factors; Wnts; Bone morphogenetic proteins; Noggin; Endothelial cells; Quail embryo

Introduction

Blood vessels in the early embryo are formed by the proliferation and differentiation of endothelial precursors, the angioblasts, in a process called vasculogenesis. Angiogenesis is the growth and sprouting of new vessels from existing ones. The growth and maintenance of the blood and lymphatic vascular systems is to a large extent mediated by members of the vascular endothelial growth factor (VEGF) family via their tyrosine kinase receptors (VEGFRs) that are expressed in angioblastic and endothelial cells (Ferrara, 2000). VEGF supports proliferation and survival of endothelial cells and induces the expression of antiapoptotic proteins in endothelial cells (Alon et al., 1995; Benjamin et al., 1999). Endothelial tyrosine kinase receptors are of fundamental importance for the transmission of both differentiation and angiogenic signals from the environment to the endothelium (Wilting et al., 2003). VEGFs bind with high affinity to five receptors: three receptor tyrosine kinases called VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (Flt-4), as well as two non-kinase receptors, neuropilin-1 (NRP-1) and NRP-2.

In the quail, the homologue of VEGFR-2 has been cloned and named Quek1 (Eichmann et al., 1993, 1997). Quek1 possesses 69% and 71% identity to murine Flk-1 and human KDR. VEGFR-2 expressing cells isolated from chick blastoderm and cultured *in vivo* can give rise to both endothelial and hematopoietic cells (Eichmann et al., 1997). VEGFR-2 deficient mice failed to form yolk-sac blood islands and lacked organized blood vessel formation in the embryo proper (Shalaby et al., 1995). Thus, embryonic blood vessel formation depends on this receptor of which the expression has been studied in detail (Yamaguchi et al., 1993; Quinn et al., 1993; Eichmann et al., 1993; Wilting et al., 1997; Nimmagadda et al., 2004). In the quail paraxial mesoderm, expression of *Quek1* can be observed in the lateral portion of both the segmental plate mesoderm and the epithelial somite. Initial expression in the somite is restricted to the dorsolateral quadrant (Eichmann et al., 1993; Nimmagadda et al., 2004; Wilting et al., 1997). Later, a medial expression domain is established in the sclerotome adjacent to the neural tube. In the avascular notochord, *Quek1* is

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being expressed from day 4 onwards (Eichmann et al., 1993; Nimmagadda et al., 2004; Wilting et al., 1997). The mechanisms controlling VEGFR-2 expression can give us a key to understand the regulation of these expression patterns.

The lateral plate is subdivided into somatic and splanchnic mesoderm by the coelomic cavity. By means of quail/chick transplantation experiments, it has been shown that both somitic and splanchnic mesoderm have the potential to give rise to endothelial progenitors (Wilting et al., 1997; Pardanaud and Dieterlen-Lievre, 1999; Pardanaud et al., 1987). Since angioblasts do not cross the embryonic midline (Klessinger and Christ, 1996), it has been suggested that notochord-derived signals inhibit midline crossing and negatively regulate blood vessel formation. In contrast, 3 to 5 days after grafting quail segmental plate to chick, $QH1^+$ vascular plexuses were found around and inside the neural tube. Further some QH^+ cells had crossed the midline either as single cells or organized into vascular structures (Pouget et al., 2006). Our previous observations demonstrated that the BMP4 signaling pathway is involved in the regulation of *Quek1* expression (Nimmagadda et al., 2005). FGF8 is found to be expressed in the intermediate mesoderm along with BMP4. Further, FGF and BMP signaling have been found to induce cardiac differentiation (Lough et al., 1996; Alsan and Schultheiss, 2002). Fibroblast growth factors (FGFs) induce proliferation and differentiation of epithelial and mesenchymal cells. Several FGFs have previously been found to be produced by tumor cells and induce angiogenesis (MacArthur et al., 1995; Tanaka et al., 1995; Johnson et al., 1998; Marsh et al., 1999; Dorkin et al., 1999; Gerwins et al., 2000). Several Wnt receptors and transcriptional effectors are expressed in human and mouse endothelial cells and also in many tumor types. Wnt/β-catenin signaling has been shown to promote endothelial cell proliferation whereas suppression of Wnt activity reduces Flk^+ embryonic stem cells (Masckauchan et al., 2005; Wang et al., 2006).

In the present study, we extended our previous work (Nimmagadda et al., 2005) on the regulation of *Quek1* expression in the somite. We found that apart from the inhibiting effect of the notochord and the inducing role of BMP4 on *Quek1* expression, additional signaling mechanisms are involved in this process. We show that FGF8 from the intermediate mesoderm and Wnts (1 and 3a) from the neural tube are involved in the induction of *Quek1* expression. We also show that over-expression of FGF8 or Wnt-1 or 3a induces the formation of additional endothelial cells whereas SU 5402 and Sfrp-2 inhibits *Quek1* expression leading to a loss of endothelial cells. These results demonstrate that the regulation of *Quek1* expression in somites is mediated by cooperative actions of BMP, FGF and Wnt-signaling pathways.

Materials and methods

Preparation of quail embryos

Fertilized quail eggs (*Coturnix coturnix japonica*) were incubated at 38 °C under 80% humidity and the embryos were staged according to Hamburger and Hamilton (1952). Experiments were performed on embryos at stages 12–14.

Implantation of beads

(a) Purified recombinant mouse FGF-8b and 8c protein (R&D Systems) were diluted in PBS to a concentration of 1 μg/μl. For application of FGF, Affigel beads of approximately 80–120 μm in diameter (BioRad Laboratories) were rinsed in PBS and incubated with FGF-8b or 8c protein solution for overnight at 4 °C. Beads soaked in PBS were used as controls.
(b) FGF signaling inhibitor SU 5402 (Calbiochem) was dissolved in dimethyl sulfoxide to a concentration of 10 mM. For application of SU 5402, AG 1-X2 Resin carrier beads (diameter: 100 μm; BioRad Laboratories) were incubated for overnight in SU 5402 solution. Control beads were incubated in dimethyl sulfoxide.

For bead implantation, paraxial mesoderm (somite 14–19 of HH-stage 12–13 embryo) was punctured with an electrolytically sharpened tungsten needle, and a bead was inserted into the mesenchyme using a blunt glass needle. Embryos were reincubated for 16–20 h, processed for whole mount *in situ* hybridization. None of the controls had an effect on *Quek1* expression.

Cell injection

Wnt3a-, Wnt1-, Wnt4- and Sfrp2-expressing cells were a gift from Andreas Kispert (Medizinische Hochschule Hannover, Germany). Cell lines were cultured as described elsewhere (Lamb et al., 1993). Confluent cultures were harvested, cells were washed in phosphate-buffered saline (PBS), pelleted and resuspended in a minimal volume of medium. For cell injection, the ectoderm (at the level of somite I–V of HH stage 13–14 embryos) was punctured with a tungsten needle. With the help of a blunt glass needle, a tunnel was made below the ectoderm and concentrated cell suspensions were locally applied with a micropipette along the length of the tunnel. Embryos were reincubated from 16 to 20 h, processed for whole-mount *in situ* hybridization. Control cells showed no effect on target genes expression (not shown).

In situ hybridization

Embryos were fixed overnight at 4 °C in 4% PFA. Embryos were washed twice in PBT, dehydrated in methanol and stored at 4 °C. Whole mount *in situ* hybridization was performed as previously described (Nieto et al., 1996). Selected stained embryos were embedded in 4% agar and sectioned with a Leica Vibratome at 50 μm. For Quek1, we used the cloned Quek1 4500-bp fragment as template. Linearization was performed with *Hin*dIII and *Sph*I (Quek1/VEGFR-2) to produce antisense and sense probes.

Immunohistochemistry on whole mounts for the detection of quail endothelial cells

Selected embryos after *in situ* hybridization were used for immunohistochemistry, fixed overnight in 4% paraformaldehyde (PFA), washed in PBS. Following a brief wash in PBS, embryos were sectioned, incubated overnight with monoclonal QH1 antibody (DSHB; 1:5 in PBS). After extensive washing in PBS, embryos were incubated overnight in secondary antibody (Cy3-conjugated goat anti-mouse IgG antibody; Jackson ImmunoResearch, 1:100 in PBS). Subsequently, sections were washed in PBS, mounted in Mowiol (Merk) and analyzed with an epifluorescence microscope (Axiophot; Zeiss).

Results

FGF8 signals from the intermediate mesoderm (IM) induce Quek1 expression in the somite

Expression of *Quek1* is restricted to the lateral portion of the somite (Figs. 1A–B). Previous studies have shown that *BMP4* expressed in the IM and/or LPM is required for induction of